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(54) Title: INHIBITION OF PLANT CELL RESPIRATION

(57) Abstract

A variety of genes may be used to express protein which inhibits full expression of selected characteristics of plants. These inhibit functions which are critical to full expression of a genetic characteristic. Also known as "killer" genes, a particular area of interest is in the expression of a protein inhibitor of mitochondrial function leading to cell death and failure to produce viable pollen, thus imparting male sterility. When inserted as a module in a gene cascade which permits external control of expression, male fertility may be restored.

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Inhibition of Plant Cell Respiration

The present invention relates to a method of inhibiting respiration of a plant cell by use of a gene, which is expressible in plants, to inhibit mitochondrial function, hence disrupting full expression of a selected plant characteristic.

According to the present invention there is provided a method of inhibiting gene expression in a target plant tissue comprising stably transforming a plant cell of a type from which a whole plant may be regenerated with a gene construct carrying a tissue-specific or a development-specific promoter which operates in the cells of the target plant tissue and a disrupter gene encoding a protein which is capable, when expressed, of inhibiting respiration in the cells of the said target tissue resulting in death of the cells.

Preferably the disrupter gene is selected from:

(a) The mammalian uncoupling protein (UCP) cloned from mammalian (usually rat) brown adipose tissue.

(b) A mutated form of the gene for the β-subunit of F_1-ATPase which has sequences added or deleted such that these changes result in the retention of the ability to assemble with other subunits but interfere with function as an ATP synthase. The ability of these altered subunits to assemble correctly will be important as the required phenotypic effect of their expression will depend on their competition with wild-type subunits for binding sites in the enzyme complex. Thus complexes containing non-functional subunits will only be weakly active and mitochondria harbouring these complexes will be non-functional.

(c) A mutated, synthetic form of the oli 1 gene encoding subunit 9 of the F_o-ATPase. Mutations created as described at (b) above.

(d) A mutated form of a mitochondrial transit pre-sequence which malfunctions during transfer resulting, probably by blocking of receptor sites, in the disruption of protein transport to mitochondria.

(e) Gene constructs involving a fusion between the β-subunit gene from yeast and the β-glactosidase gene from E. coli, resulting in expression of a disrupting fusion protein.

Preferably the promoter is a tapetum-specific promoter or a pollen-specific promoter, so that on expression of the said disrupter protein therein the regenerated plant is in male sterile. More preferably the said tapetum-specific promoter has the sequence shown in Figure 1 or 2 or 3 of the accompanying drawings.

Plasmids containing the DNA sequences shown in Figures 1, 2 and 3 have been deposited under the terms of the Budapest Treaty, details being as follows:

Plasmid pMS10 in an Escherichia coli strain RR1 host, containing the gene sequence shown in Figure 1 herewith, and deposited with the National Collection of Industrial & Marine Bacteria on 9th January 1989 under the Accession Number NCIB 40090.

Plasmid pMS14 in an Escherichia coli strain DH5α host, containing the gene control sequence shown in Figure 2 herewith, and deposited with the National Collection of Industrial & Marine Bacteria on 9th January 1989 under the Accession Number NCIB 40099.

Plasmid pMS18 in an Escherichia coli strain RR1 host, containing the gene control sequence shown in Figure 3 herewith, and deposited with the National

Collection of Industrial & Marine Bacteria on 9th January 1989 under the Accession Number NCIB 40100.

The isolation and characterisation of these gene control sequences of this invention are described in full in a copending patent application.

The present invention also provides a plant having stably incorporated in its genome by transformtion a gene construct carrying a gene construct carrying a tissue-specific or a development-specific promoter which operates in the cells of the target plant tissue and a disrupter gene encoding a protein which is capable, when expressed, of inhibiting respiration in the cells of the said target tissue resulting in death of the cells.

The invention also provides a plant, particularly a monocotyledonous plant, and more particularly a corn plant, having stably incorporated within its genome a gene construct carrying a tissue-specific promoter which operates in the cells of the said target tissue and a disrupter gene encoding a protein which is capable of inhibiting respiration in the said cells of the said target tissue resulting in death of the cells.

These gene constructs may be used as a means of inhibiting cell growth in a range of organisms from simple unicells to complex multicellular organisms such as plants and animals. By the use of tissue- or cell-specific promoters, particular cells or tissue may be targeted and destroyed within complex organisms. One particular application intended for this invention is in the destruction of cells essential for male flower development, leading to male sterility.

The invention therefore provides a method of preventing or inhibiting growth and development of plant cells based on gene constructs which inhibit respiratory function. The technique has wide application in a

number of crops where inhibition of particular cells or tissue is required.

Of particular interest is the inhibition of male fertility in maize for the production of F1 hybrids in situ. The concept of inhibition of mitochondrial function as a mechanism for male sterility arises from some previous research on T-type cytoplasmic male sterility in maize (cms-T) which has shown an association between the male sterile phenotype and mitochondrial dysfuction. Although a direct causal relationship has yet to be established between mitochondrial dysfunction and cms-T, an increasing body of evidence suggests that fully functional mitochondria, particularly in the tapetal cells, are essential. This is particularly critical during microsporogenesis since the metabolic demands placed on the tapetal cells results in a 40-fold increase in mitochondrial number.

Thus we provide a number of negative mutations which act upon mitochondria to uncouple oxidative phosphorylation. When specifically expressed in maize anther tissue these mutations will result in a male sterile phenotype.

The proposed disrupter protein, UCP, is instrumental in the thermogenesis of mammalian brown adipose tissue and exists as a dimer in the mitochondrial inner membrane forming a proton channel and thus uncoupling oxidative phosphorylation by dissipation of the proton electrochemical potential differences across the membrane.

An alternative is the use of chimeric gene constructs in which domains are swapped, creating non-functional proteins. The target proteins here are the β-subunit of F_1-ATPase and subunit 9 of the F_0-ATPase. During assembly of functional ATPase complexes,

the altered chimeric subunits will complete for binding sites normally occupied by the naturally occurring subunits, particularly when the chimeras are over expressed compared with the endogenous genes. Mitochondrial function will be disrupted since F_1 and F_0 ATPase's assembled with altered subunits are likely to be weakly active or non-functional.

The method employed for transformation of the plant cells is not especially germane to this invention and any method suitable for the target plant may be employed. Transgenic plants are obtained by regeneration from the transformed cells. Numerous transformation procedures are known from the literature such as agroinfection using Agrobacterium tumefaciens or its Ti plasmid, electroporation, microinjection of plant cells and protoplasts, microprojectile transformation and pollen tube transformation, to mention but a few. Reference may be made to the literature for full details of the known methods.

The development and testing of these gene constructs as disrupters of mitochondrial function in the unicellular organism, yeast, will now be described. A mechanism by which these gene constructs may be used to inhibit plant cell growth and differentiation in transformed plants will also be described. The object of these procedures is to use yeast as a model system for the identification and optimisation of gene constructs for expressing proteins which disrupt mitochondrial function. Plant cells will then be transformed with the selected constructs and whole plants regenerated therefrom.

The accompanying drawings are as follows:

Figure 1 shows the DNA sequence of a tapetum-specific promoter, carried by plasmid pMS10;

Figure 2 shows the DNA sequence of a tap tum-specific promoter, carried by plasmid pMS14;

Figure 3 shows the DNA sequence of a tapetum-specific promoter, carried by plasmid pMS18;

Figure 4 is a map of plasmid pCGS110-UCP;

Figure 5 shows the mRNA sequence of mammalian uncoupling protein gene from plasmid pCGS110-UCP (shown in Figure 4);

Figure 6 is a flowchart representation of the generation of a leu2, gal1 yeast strain;

Figure 7 is a table showing the effect of addition of galactose on the growth of BET9 and BET27 transformants;

Figure 8 shows results of growth curve analysis of BET9 (Figure 8A) and BET27 (Figure 8B) transformants grown on gly/cas medium over a period of 65 hours in the presence or absence of galactose;

Figure 9 is the growth curve analysis of rat UCP in strain BET9 grown on gly/cas medium over a period of 50 hours in the presence or absence of galactose;

Figure 10 is the growth curve analysis of rat UCP in strain BET9 grown on raffinose medium over a period of 45 hours in the presence or absence of galactose;

Figure 11 illustrates the construction of plasmid YIP/UCP from pKV49-UCP and YIp5;

Figure 12 is a map of plasmid pGR208 (Figure 12A) and the sequence of oligonucleotides used to mutate the β-subunit gene of F_1-ATPase (Figure 12B);

Figure 13 shows maps of plasmids pKV49 (Figure 13A) and pKV49-UCP (Figure 13B);

Figure 14 shows schematically the construction of a β-subunit/β-galactosidase fusion protein;

Figure 15 is a plasmid map of pKV49/BLZ;

Figure 16 is a plasmid map of pMS10-5; and,

Figure 17 is a plasmid map of pBin/MS10-UCP.

The invention will now be illustrated by the following Exampl s.

EXAMPLE 1

It was known from reports in the literature that the rat UCP gene inserted in the yeast/E. coli shuttle vector gave only low levels of expression of UCP. The yeast was Saccharomyces cerevisiae strain YM147 and the UCP gene was available on plasmid pCGS110-UCP.

Given the lack of useful expression levels with the wild type gene, modification of the rat UCP gene using site directed mutagenesis was carried out. the following modifications were made:

1. Introduction of a BamHI site seven nucleotides 5′ to the AUG methionine initiation codon;
2. Modification of the sequence around the AUG methionine initiation codon to conform to the yeast consensus sequence ATAATG;
3. Deletion of an internal BamHI site; and,
4. Introduction of a BamHI site one nucleotide 3′ to the TAG termination codon.

These modifications result in the deletion of the untranslated 5′ and 3′ rat UCP sequences as well as the introduction of a yeast consensus sequence at the methionine initiation codon.

The 1.9 kb EcoRI/PstI fragment from the plasmid pCGS110-UCP (a map of the plasmid is shown in Figure 4 and the mRNA sequence of the UCP gene is shown in Figure 5) carrying the GAL10 promoter region and the rat UCP cDNA was cloned into the EcoRI/PstI sites of M13mp19 DNA. Sequencing of the resultant construct was carried out to ensure the correct structure.

Site directed mutagenesis was carried out according to the directions given in the Amersham (Trade Mark) mutagenesis kit using three different

oligonucleotides as follows:

UCP-1	wild type	CTCTGCCCTCCGAGCCAAGATGGTGAGTT
	mutant	CTCTGCCCTCGGATCC(ATAATG)GTGAGTT
UCP-2	wild type	TGCGACTCGGATCCTGGAACG
	mutant	TGCGACTCGGTTCCTGGAACG
UCP-3	wild type	ACCACATAGGCGACTTGGAG
	mutant	ACCACATAGGATCCGACTTGGAG

Oligonucleotide UCP-1 was used to introduce the yeast consensus sequence (bracketed) which occurs around the methionine initiation codon, as well as the introduction of the BamHI cleavage site (underlined).

Oligonucleotide UCP-2 was used to delete an internal BamHI site (underlined).

Oligonucleotide UCP-3 was used to introduce a BamHI site immediately after the TAG stop codon (underlined).

These three mutations allowed the isolation of the entire UCP coding sequence on a 0.93 kb BamHI fragment.

After selection of mutant clones the modified DNA was digested with BamHI. Three clones from twenty selected gave inserts of 0.93 kb upon digestion with BamHI.

Sequencing of the clones UCPS1 and UCPS4 revealed that the UCP gene had been correctly modified with no unwanted changes present. The UCP gene was then transferred to the yeast expression plasmid pKV49 which allows expression of foreign genes in S. cerevisiae under the control of the strong PGK promoter and the GAL1-10 UAS allowing induction/repression of the foreign gene according to whether or not galactose is present in the growth medium. The 0.93 kb BamHI fragment containing the modified UCP gene was cloned into pKV49 at the BglII restriction site, resulting in the construct pKV49-UCP.

TRANSFORMATION OF YEAST WITH pKV49-UCP CONSTRUCT

a) Development Of Suitable Yeast Strain

For a recipient for the pKV49-UCP construct we needed a yeast strain carrying the appropriate markers for transformation and allowing induction of gene expression from the GAL1-10 UAS while being unable to utilise glactose as a carbon and energy source (GAL1, GAL2). Such strains were generated by mating yeast strains YM147 and SF747. After selection of diploids on minimal plates containing uracil, the colonies were transferred to sporulating media. The resulting spores were grown on YDP plates prior to the resulting yeast colonies being characterised (Figure 6). Two new yeast strains BET9 (ura3, trpl1, leu2, his3, gal1) and BE27 (ura3, trp1, leu2, gal1) were isolated, both of which are suitable for transformation with PkV49 based constructs.

b) Yeast Transformation

Yeast strains BET9 and BE27 were transformed with pKV9 and pKV49-UCP DNA; transformants were selected using the appropriate auxotrophic selection (leu) and checked by plasmid isolation followed by restriction mapping. Single colonies from each of the four different transformants BET9/pKV49, BET9/pKV49-UCP, BE27/pKV49 and BE27/pKV49-UCP) were resuspended in sterile water prior to being spotted onto plating media containing a variety of carbon sources (Figure 7) both in the presence and absence of galactose. Results from these plate tests (Figure 7) indicated that on a few of the carbon sources used, the presence of both galactose and the pKV49-UCP construct resulted in poorer growth of the resulting yeast colonies. The greater effect on retardation of growth was observed with the glycerol/casamino (gly/cas) medium containing glactose

for both pKV49-UCP transformants. Transformants either lacking the UCP gene or induced by galactose grew at the same rate as the untransformed BET9 and BE27 strains.

GROWTH CURVE ANALYSIS

As plating tests had indicated poor growth of pKV49-UCP transformants on gly/cas medium in the presence of galactose, growth curve analysis in liquid culture was carried out to determine more accurately the magnitude of the growth defect. The results in Figure 8 substitute the results of plating tests and indicate that neither the presence of pKV49-UCP DNA or galactose alone is sufficient to have any effect on the yeast cell growth rates, while the presence of both severely retards growth. As our initial results using the yeast strain YM147 transformed with the construct pCGS110-UCP had not shown any significant growth defect on any of the tested carbon sources in the presence of galactose, it would appear that the modification of the UCP gene and/or the use of a different vector (pKV49) have resulted in an observable growth defect.

ANALYSIS OF UCP EXPRESSION

As the growth curve analysis had indicated no detectable differences between the BE27 and BET9 transformants (Figure 8), it was decided only to use the BET9 transformants in subsequent experiments. Repeat growth analysis on gly/cas medium both in the presence and absence of galactose was carried out with the BET9 transformants. Cultures were allowed to grow for 47 hours to ensure that the same growth curve characteristics observed previously (Figure 9) were repeated. Cells were then harvested, total cell proteins were isolated and fractionated (in duplicate) by SDS-PAGE on a 10% polyacrylamide gel. One set of fractionated proteins were stained with Coomassie Blue

to ensure equal loading of the prot ins while the other set wer transferred to nylon membrane and subjected to Western blot analysis using the rat UCP antibody. The Western blot showed two main features:

1) The comparative level of UCP expression between the BET9/pKV49-UCP transformant and the VY147/pCGS110-UCP transformant reveals that the UCP expression has increased approximately 50-100 fold as a consequence of our modifications.

2) The yeast transformant which exhibits defective growth when grown on gly/cas medium in the presence of galactose also expresses substantial amounts of UCP.

It can be concluded from these results that the modification of the UCP gene and/or its subsequent cloning into the pKV49 vector has resulted in the increased level of UCP expression relative to the levels initially detected with the pCGS110-UCP construct. Growth curve analysis indicates that the expression of UCP has an effect on the growth rates of yeast cells grown under certain conditions. As yet we have not been able to identify the specific effect that the increased levels of UCP expression have on yeast cell growth rates but preliminary results implicate a mitochondrial defect.

Growth curve analysis carried out in the raffinose medium (a fermentable carbon source which should not affect Gal regulation) of the BET9 transformants grown in the presence or absence of galactose indicate that the presence of both the UCP gene and galactose has no effect on growth rates (Figure 10). Western blot analysis of the proteins isolated from cells harvested during these growth curves reveals levels of UCP expression similar to those found in cells grown in

gly/cas medium in the presence of galactose.

The UCP det cted in BET9/pKV49-UCP transformants grown without added galactose is probably due to galactose residues released into the medium by hydrolysis of raffinose, possibly during the autoclaving. These observations indicate that the presence of UCP in yeast cells grown on a fermentable carbon source (no requirement for oxidative phosphorylation) has no effect on cell growth rates, while cells growing on the gly/cas medium (a non-fermentable carbon source) expressing UCP exhibit defective growth.

LOCATION OF UCP IN YEAST CELLS

Rat UCP is a major component of the mitochondrial inner membrane of brown adipose tissue. Unlike many other polypeptides found in the inner membrane it does not contain a cleavable signal sequence, the targeting information being encoded internally within the amino acid sequence of the protein. As our results indicate that the expressed UCP has an effect on the rate of yeast cell growth then it is important to determine the precise location of the protein expressed in yeast cells. Initial Western blot analysis of total mitochondrial proteins shows the UCP expressed by the pKV49-UCP transformant to be located in the mitochondrial fraction.

Subsequent mitochondrial fractionation revealed that the majority of the UCP is located in the inner membrane fraction of yeast mitochondria. Although some of the UCP appears to be located in the inner-membrane space, this observation is most likely due to contamination of this fraction with some of the inner membrane fraction. Similar results have been obtained with the location of the β-subunit of the F_1-ATPase

complex from yeast cell mitochondria. The β-subunit which is a component of the inner membrane is also detected in our inter-membrane space preparations. However, these results do show that the targeting information within the rat UCP is sufficient to target the UCP to the inner membrane of yeast mitochondria where it could function a an uncoupler protein.

UCP TRANSCRIPT ANALYSIS

RNA has been isolated from many of the growth curve experiments described previously. We are currently carrying out Northern blot analysis in order to determine whether the patterns of UCP expression are reflected by the UCP transcript signals

EFFECT OF COPY NUMBER ON UCP EXPRESSION

The transformation of yeast cells with shuttle vectors containing the origin of replication from the yeast 2 μm circle, such as pKV49-UCP, results in these plasmids being present at approximately 40-50 copies per cell. Consequently any foreign gene carried by the plasmid will be present at the same relatively high copy number which may result in the expression of the foreign protein at a higher level than would be seen for a gene present at a low copy number. We have therefore attempted to lower the copy number of the UCP gene by integrating it into the yeast chromosome at a single site resulting in a genetically stable, single-copy transformant. The vector YIp5 (Figure 11) is an integrating yeast vector carrying the ura3 gene; it is unable to replicate autonomously in yeast.

The 1.8 kb EcoRI/SalI fragment from pKV49-UCP containing the rat UCP gene along with the PGK promoter and GAL UAS was cloned into the EcoRI/SALI sites of YIp5 DNA. The resultant plasmid UIP-UCP (Figure 11) was checked by restriction enzyme mapping to ensure the UCP

gene was correctly inserted . The YIP-UCP plasmid was cut with the restriction enzyme EcoRV (which cuts in the middle of the URA3 gene (Figure 11) and the linearised YIP/UCP DNA was used to transform the yeast cell lines BET9 and BE27. Transformants were initially screened on minimal plates by selecting for uracil prototrophy and after 7-10 days two transformants from each cell line were streaked out onto YPD plates (non-selective). The transformants were then subjected to four consecutive periods of growth on non-selective medium. One hundred colonies from each of the original four transformants were then replica plated onto both non-selective (YDP) and selective media (minimal plants + ura). All colonies grew on the selective media indicating that the URA3 gene, which is genetically linked to the UCP gene (Figure 11), had been integrated to the yeast cell chromosome. Chromosomal DNA was isolated from each of the four transformants, digested with the restriction enzyme EcoRI and fractionated on a 0.08% agarose gel. Southern blot analysis using a labelled UCP probe indicated that the UCP gene is present in the yeast chromosome of all four transformants. Western blot analysis using the rat UCP antibody will show the level of UCP expression in these transformants. Growth curve analysis of these transformants grown in the presence of galactose shows that they may have growth inhibition consistent with a mitochondrial defect.

EXAMPLE 2

MODIFICATION OF THE β-SUBUNIT OF F_1 ATPASE

The second approach we have taken to introducing mutations affecting mitochondrial function is the directed modification of functional mitochondrial proteins which when expressed in yeast might be expected to interfere with the generation of ATP. The protein

chosen for this approach is the β-subunit of the F_1-ATPase complex. The DNA sequence of the yeast β-subunit gene is known and the gene has been independently cloned and sequenced in our laboratory 918).

The F_1ATPase portion of ATP synthase catalyses the terminal step of oxidative phosphorylation F_1 is an assembly of five different polypeptides designated α, β, γ, δ and ε. Experiments carried out by Parsonage et al on modification of the β-subunit of F_1-ATPase from E. coli identified specific amino acid residues of the β-subunit that appear to be very important for catalysis of both ATP synthesis and hydrolysis. Two mutations in particular were shown to result in greatly impaired catalysis without causing major structural perturbation of the F_1-ATPase. One of these mutations resulted from changing the strongly conserved lysine residue occurring in the catalytic nucleotide-binding domain at position 155 to a glutamine residue while the other mutation resulted from changing the methionine residue at position 209 to a leucine residue. Both of these mutations have been reposed to exert their effect by the prevention of confirmational changes required from the catalytic cooperativity in the F_1 complex.

As the assembly of these mutated β-subunit proteins into the F_1-ATPase is not affected, then it was felt that similar mutants of the β-subunit in yeast might compete for assembly into F_1-ATPase. It was thought that the result of having both wild-type and mutated β-subunits in the same F_1-ATPase would perhaps result in impaired catalysis resulting in a decrease in ATP production and retarded cell growth.

The β-subunit of F_1-ATPase from a wide variety of sources has been shown to by highly conserved at the

amino acid sequence and comparison of the S cerevisiae β-subunit amino acid sequence with that from E. Coli confirms that the lysine and methionine residues shown by Parsonage et al to be very important for catalytic activity are conserved, with the lysine and methionine residues occurring at positions 196 and 255 respectively on the yeast β-subunit sequence.

In order to carry out SDM the wild-type β-subunit gene from yeast was isolated from the plasmid pGR208 (Figure 12) as an EcoRI/BamHI fragment which was cloned into M13mp19. Two mutated β-subunit genes were constructed: mutant BB1 has both the Met255 and Lys196 converted to isoleucine and glutamine respectively while mutant BB2 has only the lysine to glutamine mutation (Figure 12). Following sequence analysis to ensure correct mutagenesis with no unwanted mutations, the mutated β-subunit genes were removed from mp19 by EcoRI/BamHi digests. The fragments containing the genes were then blunt-ended and ligated to BglII digested pKV49 (Figure 13) which had previously been blunt-ended. We have both mutated β-subunit genes cloned into pKV49 (pKV49-BB1 and pKV49-BB2) and have transformed the yeast strain BET9 with both these constructs. Growth curve and plate growth from both mutated β-subunit transformants show tht the transformants have altered growth characteristics which are consistent with a mitochondrial defect.

Concurrently with the transformation of strain BET9 with the mutated β-subunit genes, gene disruption may be used to construct a derivative of strain BET9 which will fail to synthesize β-subunit. The resultant strain will therefore be unable to grow on non-fermentable carbon sources although it will be easily maintainable on a fermentable carbon source such as glucose.

Transformation of this strain with plasmids bearing the mutated β-subunit genes, followed by measuring the transformants' growth characteristics on a non-fermentable carbon source, shows that the altered β-subunit is unable to support oxidative phosphorylation.

EXAMPLE 3

FUSION PROTEINS

An alternative strategy for selectively perturbing mitochondrial function is the expression of a fusion protein which results in either poor or no yeast cell growth. The candidate fusion protein chosen from this project contains the N-terminal region of the yeast ATP synthase β-subunit fused to most of β-galactosidase from E. coli and has been constructed by gene fusion (Figure 14). This β-subunit/β-galactosidase fusion protein has already been shown to be targeted to the inner membrane of yeast mitochondria 921) and cells expressing this fusion protein appear to be unable to grow on a non-fermentable carbon source. In the presence of the fusion protein the transducing capacity of the mitochondrial membrane as measured by the ^{32}P-ATP exchange reaction is only 9% of that measured in the absence of the fusion protein. As yet the mechanism of this description has not been evaluated but the gene fusion is thought to produce a protein which becomes trapped in the inner membrane and interferes with function(s) essential for respiratory growth.

CONSTRUCTION OF THE ATP2/LacZ GENE FUSION

The plasmid pGR208, which contains the yeast ATP2 DNA encoding ATP synthase β-subunit gene (Figure 12), was digested with EcoRI plus BamHi resulting in the release of a 1.1kb fragment coding for the first 350 amino acids of the β-subunit protein. pMUR1720 is a

pUC8 based plasmid which contains a LacZ gene contained within an EcoRI/NarI fragment (Figure 14). Cloning of the 1.1kb EcoRI/BamHi DNA fragment coding for the first 350 amino acids of the yeast β-subunit protein into the EcoRI/BamHi sites of pMUR1720 (Figure 14a) results in an in-frame fusion between the 350 amino acids of the β-subunit and the entire (minus the first eight amino acids) LaZ protein (Figure 14). The entire β-subunit/LacZ gene fusion is now contained on the 4.3kb EcoRI/NarI fragment in construct pMUR1720-BLZ (Figure 14). This 4.3kb EcoRI/NarI fragment is currently being cloned into the pKV49 vector resulting in the pKV49-BLZ construct (Figure 15) which can be used to transform the yeast strains BET9 and BE27 and show that when induced by galactose growth defects consistent with mitochondrial inhibition arise.

EXAMPLE 4

Construction of a promoter fusion between the MS10 gene and the UCP gene

The 1830 bp HindIII to BamH1 fragment from pMS10 was ligated into the binary plant transformation vector Bin19 previously cut with HindIII and BamH1.

Following ligation the resultant plasmid was cut with BamH1 and ligated to the 930 bp UCP BamH1 fragment from plasmid pUC/UCP (s derivative of pUC19 contyaininbg the modified UCP gene cloned at the BamH1 site) to construct a fusion between the MS10 gene promoter and the UCP gene. Finally the nos 3′ terminator obtained as a 250 bp Sst1- EcoR1 fragment from vector pTAK1 wasligated into the MS10-UCP construct previouslt cut with Sst1 and EcoR1.

The resulting plasmid is termed pBin/MS10-UCP and contains the MS10 promoter, the UCP gene, nos 3′ terminator expression cassette located between the right

and left border sequences of Agrobacterium T-DNA allowing effici nt transformation into tobacco cells.

EXAMPLE 5

Transformation of tobacco plants with pBin/MS10 promoter gene constructs

The recombinant vector pBin/MS10-UCP was mobilised from E Coli (TG-2) onto Agrobacterium tumefaciens (LBA4404) in a triparental mating on L-plates with E Coli (HB101) harbouring pRK2013. Transconjugants were selected on minimal medium containing kanamycin (50μg/cm^3) and streptomycin (500μg/cm^3).

L-Broth (5 cm^3) containing kanamycin at 50 g/cm^3 was inoculated with a single Agrobacterium colony. The culture was grown overnight at 30°c with shaking at 150 rpm. This culture (500μ1) was inoculated into L-Broth containing kanamycin (50μ g/cm^3) and grown as before. Immediately before use the Agrobacteria were pelleted by spinning at 3000 rpm for 5 minutes and suspended in an equal volume of liquid Murashige and Skoog (MS) medium.

Feeder plates were prepared in 9 cm diameter petri dishes as follows. Solid MS medium supplemented with 6-Benzyl-aminopurine (6-BAP) (1 mg/1) and 1-Naphthaleneacetic acid (NAA) (0.1 mg/1) was overlaid with Nicotiana tabacum var Samsun suspension culture (1 cm^3). One 9 cm and one 7cm filter paper discs were placed on the surface.

Whole leaves from tissue culture grown plants were placed in the feeder plates. The plates were sealed with "Nescofilm" and incubated overnight in a plant growth room (26°C under bright fluorescent light).

Leaves from the feeder plates were placed in Agrobacteria suspension in 12 cm diameter petri dishes and cut into 1- 1.5 cm^2 sections. After 20 minutes the leaf pieces were returned to the feeder plates which

were s aled and replaced in the growth room. After 48 hours incubation in the growth room the plant material was transferred to MS medium supplemented with 6-BAP (1 mg/1), NAA (0.1 mg/1), carbenicillin (500$\mu g/cm^3$) and kanamycin (100 $\mu g/cm^3$), in petri dishes. the petri dishes were sealed and returned to the growth room.

Beginning three weeks after inoculation with Agrobacterium, shoots were removed from the explants and placed on MS medium supplemented with carbenicillin (200 $\mu g/cm^3$) and kanamycin (100$\mu g/cm^3$) for rooting. Transformed plants rooted 1-2 weeks after transfer.

Following rooting, transformed plants were transferred to pots containing soil and grown in the glasshouse. Roughly one month after transfer the plants flowered.

The anthers of the tobacco plants containing the pBin/MS10-UCP construct were were assayed for expression of the UCP gene by Northern blotting of RNA samples, and the effect of UCP expression on pollen development determined.

1. A method of inhibiting gene expression in a target plant tissue comprising stably transforming a plant cell of a type from which a whole plant may be regenerated with a gene construct carrying a tissue-specific or a development-specific promoter which operates in the cells of the target plant tissue and a disrupter gene encoding a protein which is capable, when expressed, of inhibiting respiration in the cells of the said target tissue resulting in death of the cells.

2. A method according to claim 1 in which, the disrupter gene is the mammalian uncoupling protein (UCP) gene.

3. A method according to claim 1 in which, the disrupter gene is a mutated form of the gene for the β-subunit of F_1-ATPase which has sequences added or deleted such that these changes result in the retention of the ability to assemble with other subunits but interfere with function as an ATP synthase.

4. A method according to claim 1 in which, the disrupter gene is a mutated, synthetic form of the oli 1 gene encoding subunit 9 of the F_o-ATPase.

5. A method according to claim 1 in which, the disrupter gene is a mutated form of a mitochondrial transit pre-sequence which malfunctions during transfer resulting in the disruption of protein transport to mitochondria.

6. A method according to claim 1 in which, the disrupter gene is a gene construct carrying a fusion between the β-subunit gene from yeast and the β-galactosidase gene from E. coli, resulting in expression of a disrupting fusion protein.

7. A method as claimed in claim 1, in which the promoter is a tapetum-specific promoter or a pollen-specific promoter, so that on expression of the said disrupter protein therein the regenerated plant is in male sterile.

8. A method as claimed in claim 2, in which the tapetum-specific promoter has the sequence shown in Figure 1 or Figure 2 or Figure 3 of the accompanying drawings.

9. The plasmid designated pBin/MS10-UCP having the structure shown in Figure 17 of the accompanying drawings.

10. A plant transformation vector comprising Agrobacterium tumefaciens harbouring the plasmid pBin/MS10-UCP claimed in claim 9.

11. A plant having stably incorporated in its genome by transformation a gene construct carrying a gene construct carrying a tissue-specific or a development-specific promoter which operates in the cells of the target plant tissue and a disrupter gene encoding a protein which is capable, when expressed, of inhibiting respiration in the cells of the said target tissue resulting in death of the cells.

FIG. 1. 1/16

Nucleotide and deduc d amino acid sequence for male flower specific cDNA clone, pMS10.

```
                10                  20                  30                  40
                |                   |                   |                   |
GGC CTT GCC CGC TCG TTC CCC TCG CCT CCC CGG TCG CGC CGC TCC

    50                  60                  70                  80                  90
    |                   |                   |                   |                   |
CGC TGC CGC CGT GGC GAT TCC TGC CCG GCG GCG GCG CCG GGT TCA

              100                 110                 120                 130
               |                   |                   |                   |
GGT CCA CGG CGG CGG CGG CTG CGC GGG GCG GGA CCG ACT ATG GGA
                                                        Met Gly

    140                 150                 160                 170                 180
     |                   |                   |                   |                   |
CGG ACG ACA GCA AGA TCT CCC CCG ACG AGG AAT TCC TTC GAG GGC
Arg Thr Thr Ala Arg Ser Pro Pro Thr Arg Asn Ser Phe Glu Gly

              190                 200                 210                 220
               |                   |                   |                   |
TGC GAC TAC AAC CAC TGG CTC ATC ACC ATG GAC TTC CCG GAC CCC
Cys Asp Tyr Asn His Trp Leu Ile Thr Met Asp Phe Pro Asp Pro

    230                 240                 250                 260                 270
     |                   |                   |                   |                   |
AAG CCG TCG CGC GAA GAG ATG ATC GAG ACA TAC CTC CAG ACT CTC
Lys Pro Ser Arg Glu Glu Met Ile Glu Thr Tyr Leu Gln Thr Leu

              280                 290                 300                 310
               |                   |                   |                   |
GCC AAG GTC GTC GGG AGT TAT GAG GAG GCC AAG AAG AGG ATG TAT
Ala Lys Val Val Gly Ser Tyr Glu Glu Ala Lys Lys Arg Met Tyr

    320                 330                 340                 350                 360
     |                   |                   |                   |                   |
GCT TTT AGT ACG ACG ACT TAT GTT GGT TTT CAG GCT GTA ATG ACC
Ala Phe Ser Thr Thr Thr Tyr Val Gly Phe Gln Ala Val Met Thr

              370                 380                 390                 400
               |                   |                   |                   |
GAG GAA ATG TCA GAA AAA TTT CGC GGT TTG CCT GGA GTA GTT TTC
Glu Glu Met Ser Glu Lys Phe Arg Gly Leu Pro Gly Val Val Phe

    410                 420                 430                 440                 450
     |                   |                   |                   |                   |
ATT TTG CCT GAT TCA TAT CTA TAT CCA GAA ACA AAG GAG TAC GGA
Ile Leu Pro Asp Ser Tyr Leu Tyr Pro Glu Thr Lys Glu Tyr Gly

              460                 470                 480                 490
               |                   |                   |                   |
GGA GAC AAA TAT GAC AAT GGT GTC ATC ACT CCA AGA CCA CCA CCT
Gly Asp Lys Tyr Asp Asn Gly Val Ile Thr Pro Arg Pro Pro Pro
```

FIG. 1. (cont.)


```
   500          510          520          530          540
GTT CAT TAT AGC AGA CCA TCA AGA ACT GAC AGG AAC CGT AAC TAC
Val His Tyr Ser Arg Pro Ser Arg Thr Asp Arg Asn Arg Asn Tyr

         550          560          570          580
CGA GGA AAC TAC CAG GAT GGC CCT CCA CAG CAA GGA AAT TAC CAG
Arg Gly Asn Tyr Gln Asp Gly Pro Pro Gln Gln Gly Asn Tyr Gln

   590          600          610          620          630
AAC AAC CGT CCT CCA CCA GAA GGT GGT TAC CAG AAC AAC CCG CCG
Asn Asn Arg Pro Pro Pro Glu Gly Gly Tyr Gln Asn Asn Pro Pro

         640          650          660          670
CAG CAA GGA AAC TAC CAG ACA TAC CGC TCG CAG CAA GAT GGA AGA
Gln Gln Gly Asn Tyr Gln Thr Tyr Arg Ser Gln Gln Asp Gly Arg

   680          690          700          710          720
GGC TAT GCC CCA CAG CAG AAT TAT GCA CAA GGT GGT CAG GAT GGT
Gly Tyr Ala Pro Gln Gln Asn Tyr Ala Gln Gly Gly Gln Asp Gly

         730          740          750          760
AGA GGT TTT GGA AGG AAT GAT TAC ACA GAC CGT TCA GGC TAC AAT
Arg Gly Phe Gly Arg Asn Asp Tyr Thr Asp Arg Ser Gly Tyr Asn

   770          780          790          800          810
GGA CCC ACT GAT TTT CGA AGT CAA ACT CAG TAC CAA GGG CAT GTA
Gly Pro Thr Asp Phe Arg Ser Gln Thr Gln Tyr Gln Gly His Val

         820          830          840          850
AAT CCA GCT GGG CAA GGT CAA GGT TAC AAC AAC CCC CAA GAG CGT
Asn Pro Ala Gly Gln Gly Gln Gly Tyr Asn Asn Pro Gln Glu Arg

   860          870          880          890          900
ACG AAC TTC TCG CAA GGG CAG GGA GGA GGT TTT AGG CCT GGT GGT
Thr Asn Phe Ser Gln Gly Gln Gly Gly Gly Phe Arg Pro Gly Gly

         910          920          930          940
CCT TCA GCA CCT GGG TCT TAT GGC CAA CCA TCA GCA CCT GGA TCT
Pro Ser Ala Pro Gly Ser Tyr Gly Gln Pro Ser Ala Pro Gly Ser

   950          960          970          980          990
TAT GGT CAA CCT AAT ACA CTT GGT AAC TAT GGG CAG GTA CCT CCA
Tyr Gly Gln Pro Asn Thr Leu Gly Asn Tyr Gly Gln Val Pro Pro
```

FIG. 1.
(cont.)

```
          1000        1010        1020        1030
TCA GTG AAT CCT GGT GGT AAC AGA GTT CCT GGT GTG AAT CCT AGT
Ser Val Asn Pro Gly Gly Asn Arg Val Pro Gly Val Asn Pro Ser

  1040        1050        1060        1070        1080
TAT GGT GGG GAT GGC AGA CAG GGG GCT GGA CCA GCA TAT GGT GGA
Tyr Gly Gly Asp Gly Arg Gln Gly Ala Gly Pro Ala Tyr Gly Gly

          1090        1100        1110        1120
GAT AAC TGG CAA AGA GGT TCT GGT CAG TAT CCT AGC CCA GGT GAA
Asp Asn Trp Gln Arg Gly Ser Gly Gln Tyr Pro Ser Pro Gly Glu

  1130        1140        1150        1160        1170
GGA CAA GGA AAC TGG CAG GGA AGG CAG TAA GAG CTG ACG TGT TCC
Gly Gln Gly Asn Trp Gln Gly Arg Gln

          1180        1190        1200        1210
ACT GAA GAC AAG AAT GGC ACT TGA GAT TTA GAA ATC TCC ATC TGT

  1220        1230        1240        1250        1260
AAA ATA AAC GAC TGT GAT GCA TTA CTC TTT TTT TTT TTC TTG CAT

          1270        1280        1290        1300
TTG AAC TCT AAA CTT ATG GGC ATG CGT TAT TAC CAA ACT ACG GAT

  1310        1320        1330        1340        1350
GCA AAT TCA TTT TAG TTT TTT GGG CCA AAT GTT GGC ATT TTT AAA

AAA
```

FIG.2. 4/16

Nucleotide and deduced amino acid sequence for the male flower specific cDNA clone, pMS14.

```
                10                  20                  30                  40
GCA GGG GGG GGG GCA CAG CAA GCC AGC AGA GCA GAA AGC AGC CGC
Ala Gly Gly Gly Ala Gln Gln Ala Ser Arg Ala Glu Ser Ser Arg

    50                  60                  70                  80                  90
AGC CCC AGC CCC CAC AAA GAC GAA GGC AAC AAT GGC GCT AGA AGC
Ser Pro Ser Pro His Lys Asp Glu Gly Asn Asn Gly Ala Arg Ser

            100                 110                 120                 130
AGC CAC GCC CCC CGC GCA CTC CTC GCG CGT GCC TCG TCC TGC TGG
Ser His Ala Pro Arg Ala Leu Leu Ala Arg Ala Ser Ser Cys Trp

   140                 150                 160                 170                 180
TCC TCG GCG GCG GCA CCG GCC CGT CGT CGG TGC TCA GCG CGC CGG
Ser Ser Ala Ala Ala Pro Ala Arg Arg Arg Cys Ser Ala Arg Arg

            190                 200                 210                 220
GGC GCA GGA CCG GCG GCA GTG CCT GCC GCA GCT GAA CGC CTC CTG
Gly Ala Gly Pro Ala Ala Val Pro Ala Ala Ala Glu Arg Leu Leu

   230                 240                 250                 260                 270
CGG TGC CGC GCG TAC CTG GTG CCG GCG CGC CGG ACC CCA GCG CGG
Arg Cys Arg Ala Tyr Leu Val Pro Ala Arg Arg Thr Pro Ala Arg

            280                 290                 300                 310
ACT GCT GCA GCG CTG ACG CGC CGT GTG CAC GAG TGC GCC TGC AGC
Thr Ala Ala Ala Leu Thr Arg Arg Val His Glu Cys Ala Cys Ser

   320                 330                 340                 350                 360
ACC ATG GGC ATC ATC AAC AGC CTG CCC GGC CGG TGC CAC CTC GCC
Thr Met Gly Ile Ile Asn Ser Leu Pro Gly Arg Cys His Leu Ala

            370                 380                 390                 400
CAA GCC AAC TGC TCC GCT TGA AGC AGG GAC CTG GCA CGC GTG CTG
Gln Ala Asn Cys Ser Ala

   410                 420                 430                 440                 450
CAA TGG ATG GCA GGA GGG GAG AGG AAT AAG AAG TGT TTC CAT TTC

            460                 470                 480                 490
ACA GTG AGA GCA GTC GAG CTC CAA CGT TGT CGT CGT CGT CGT CTT
```

FIG.2.

(cont.)

```
    500         510         520         530         540
    |           |           |           |           |
CTT CTT TTG ATA TTC AGA CTC TGT CTT GCG GTC TAT ATC ATC AGC

          550         560         570         580
          |           |           |           |
ATA ATA ATA ATA AAA TAA GTA AAA CCA AAA AAA AAA AAA AA
```

FIG. 3. 6/16

Nucleotide and deduc d amino acid sequence for the male flower specific cDNA clone, pMS18.

```
                  10                  20                  30                  40
                   |                   |                   |                   |
ACA GCA GTA GCA AGA GGG ATA GAG CAA GGC CAC ACA CAC ACA CAC

      50                  60                  70                  80                  90
      |                   |                   |                   |                   |
ACC ACT AGG CTA GGT TAG CCT TTT AAT CGT CGT CGA GAA GCA AGA

                 100                 110                 120                 130
                  |                   |                   |                   |
AGG GCG CTG CAC CAA GCA GGC AAG CAA GAA GAG AGC CGA TCG ACC

    140                 150                 160                 170                 180
     |                   |                   |                   |                   |
GAG AGC TAG CAC GCG ATG GCG AGG TCT TGC CAA GAT GAT GGT GGC
                        Met Ala Arg Ser Cys Gln Asp Asp Gly Gly

                 190                 200                 210                 220
                  |                   |                   |                   |
GCA CGT CTG CTG GCC TTG CGC TGG CGT GTC GAC CGC CGA GGC AGG
Ala Arg Leu Leu Ala Leu Arg Trp Arg Val Asp Arg Arg Gly Arg

    230                 240                 250                 260                 270
     |                   |                   |                   |                   |
AAC ATC AAG ACC ACG ACG ACG GAG AAG AAG GAC GAC GCG GTG GTG
Asn Ile Lys Thr Thr Thr Thr Glu Lys Lys Asp Asp Ala Val Val

                 280                 290                 300                 310
                  |                   |                   |                   |
CAG CCG CAG AGG TTC CGC CCT TCG ACC GCC TCG GCG CGG CGC GTC
Gln Pro Gln Arg Phe Arg Pro Ser Thr Ala Ser Ala Arg Arg Val

    320                 330                 340                 350                 360
     |                   |                   |                   |                   |
CCC GGC GTT CGG CGG CCT CCC CGG CGG CAC GAT TCC TGG CAG CAG
Pro Gly Val Arg Arg Pro Pro Arg Arg His Asp Ser Trp Gln Gln

                 370                 380                 390                 400
                  |                   |                   |                   |
CAT TCC CGG GTT CAG CAT GCC CGG CAG CGG CAG CAG CCT ACC CGG
His Ser Arg Val Gln His Ala Arg Gln Arg Gln Gln Pro Thr Arg

    410                 420                 430                 440                 450
     |                   |                   |                   |                   |
GTT CAG CTT GCC CGG CAG CGG CAC GAT GCC CCT CTT CGG CGG CGG
Val Gln Leu Ala Arg Gln Arg His Asp Ala Pro Leu Arg Arg Arg

                 460                 470                 480                 490
                  |                   |                   |                   |
CTC CCC GGG CTT CAG CGG CTT CGG CGG CAT GCC CGG GTC GCC CAC
Leu Pro Gly Leu Gln Arg Leu Arg Arg His Ala Arg Val Ala His
```

FIG. 3.

(cont.)

```
   500           510           520           530           540
    |             |             |             |             |
CGC CGG CTC CGT CCC CGA GCA CGC CAA CAA GCC CTG AAC GCC AAC
Arg Arg Leu Arg Pro Arg Ala Arg Gln Gln Ala Leu Asn Ala Asn

           550           560           570           580
            |             |             |             |
AAG CGT GGT AGT AGA GGT GCT ACT GTT ACT GTA GTA CGT CGT CGT
Lys Arg Gly Ser Arg Gly Ala Thr Val Thr Val Val Arg Arg Arg

   590           600           610           620           630
    |             |             |             |             |
CTT CAT GCA TGC GTG GTT CGT GGT TTC CCT AGC TCC ATA CGA GCA
Leu His Ala Cys Val Val Arg Gly Phe Pro Ser Ser Ile Arg Ala

           640           650           660           670
            |             |             |             |
GTA GTT GGG CTT GCA CGT ACC GTA CGT CTA GCT AGC TAT ATA TAT
Val Val Gly Leu Ala Arg Thr Val Arg Leu Ala Ser Tyr Ile Tyr

   680           690           700           710           720
    |             |             |             |             |
GCT TGT GTT CTA CTG CTT TTT AGT TTA ATT ACC TGC CTG CAT TGG
Ala Cys Val Leu Leu Leu Phe Ser Leu Ile Thr Cys Leu His Trp

           730           740           750           760
            |             |             |             |
AGA GTT GGA TCT GTT TCA TTT GGT GGT GTT TGC TTT ACT ATT AGG
Arg Val Gly Ser Val Ser Phe Gly Gly Val Cys Phe Thr Ile Arg

   770           780           790           800           810
    |             |             |             |             |
TCA GTA TCT GTT TGT GGA GAC TTG GTG TTT AAT TTA TTT AGC CGT
Ser Val Ser Val Cys Gly Asp Leu Val Phe Asn Leu Phe Ser Arg

           820           830           840           850
            |             |             |             |
TTG TGA CTG GTT GTA GCT AGC GGT GGT GCG GTG GTG ATG TTC TTG
Leu

   860           870           880           890           900
    |             |             |             |             |
AGG CAT GAA TAA TGC TAC ATG CAT GTG ATG TAT CCA TGT TTT GTG

           910           920           930
            |             |             |
TGT GGT AAA CCT GTT GTT TGT ATA AGC TGT CCC
```

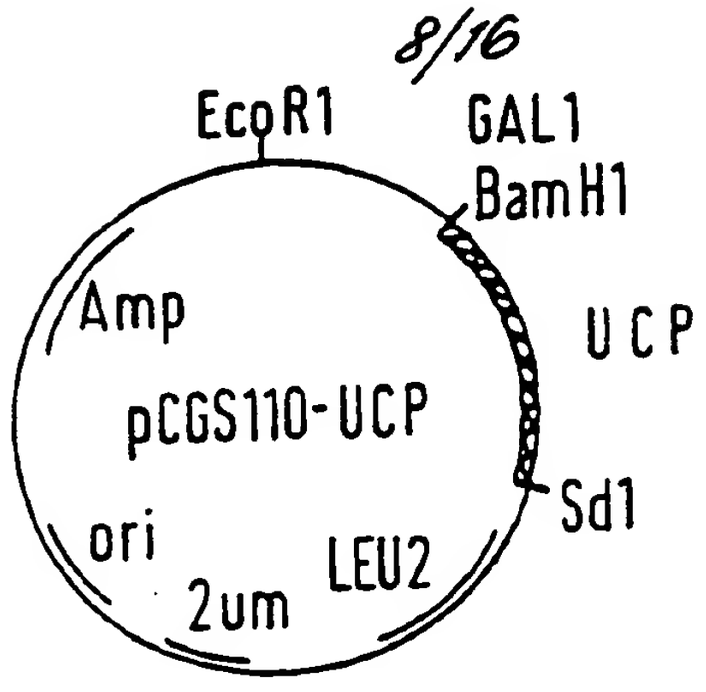


FIG. 4.

FIG. 5.

Kpn I

1 UCGAAGUUGA GAGUUCGGUA CCCACAUCAG GCAACAGUGC CACUGUUGUC

51 UUCAGGGCUG AUUCCUUUUG GUCUCUGCCC UCCGAGCCAA GAUGGUGAGU

101 UCGACAACUU CCGAAGUGCA ACCCACCAUG GGGGUCAAGA UCUUCUCAGC

151 CGGCGUUUCU GCCUGCCUAG CAGACAUCAU CACCUUCCCG CUGGACACCG

201 CCAAAGUCCG CCUUCAGAUC CAAGGUGAAG GCCAGGCUUC CAGUACUAUU

251 AGGUAUAAAG GUGUCUUAGG GACCAUCACC ACCCUGGCCA AGACAGAAGG

301 AUUGCCGAAA CUGUACAGCG GUCUGCCUGC UGGCAUCCAG AGGCAAAUCA

351 GCUUUGCUUC CCUCAGGAUU GGCCUCUACG AUACGGUCCA AGAGUACUUC

401 UCUUCAGGGA GAGAAACGCC UGCCUCUUUG GGAAGCAAGA UCUCGGCUGG

451 CUUGAUGACG GGUGGCGUGG CGGUAUUCAU UGGGCAGCCC ACAGAGGUGG

501 UGAAGGUCAG AAUGCAAGCA CAAAGCCAUC UGCACGGGAU CAAACCCCGC

551 UACACUGGGA CCUACAAUGC UUACAGAGUU AUAGCCACCA CAGAAAGCUU

601 GUCAACACUG UGGAAAGGGA CGACUCCUAA UCUAAUGAGA AAUGUCAUCA

651 UCAACUGUAC AGAGCUGGUG ACAUAUGACC UCAUGAAGGG GGCCCUUGUG

701 AACCACCACA UACUGGCAGA UGACGUCCCC UGCCAUUUAC UGUCAGCUCU

751 UGUCGCCGGG UUUUGCACCA CACUCCUGGC CUCUCCGGUG GAUGUGGUAA

801 AAACGAGAUU CAUCAACUCU CUACCAGGAC AGUACCCAAG UGUACCCAGC

851 UGUGCAAUGA CCAUGUACAC CAAGGAAGGA CCGGCAGCCU UUUUCAAAGG

901 GUUUGCGCCU UCUUUUCUGC GACUCGGAUC CUGGAACGUC AUCAUGUUUG

951 UGUGCUUUGA ACAGCUGAAG AAAGAGCUGA UGAAGUCCCG GCAGACAGUG

1001 GACUGCACCA CAUAGGCGAC UUGGAGAAAG GGAUGCUAAA CACCAUUGGG

1051 CUCCUAUGCU GGGCUCCUAU GCUGGGAGAC CACGAAUAAA ACCAUCCAAA

1101 GAAAUCAGAC G

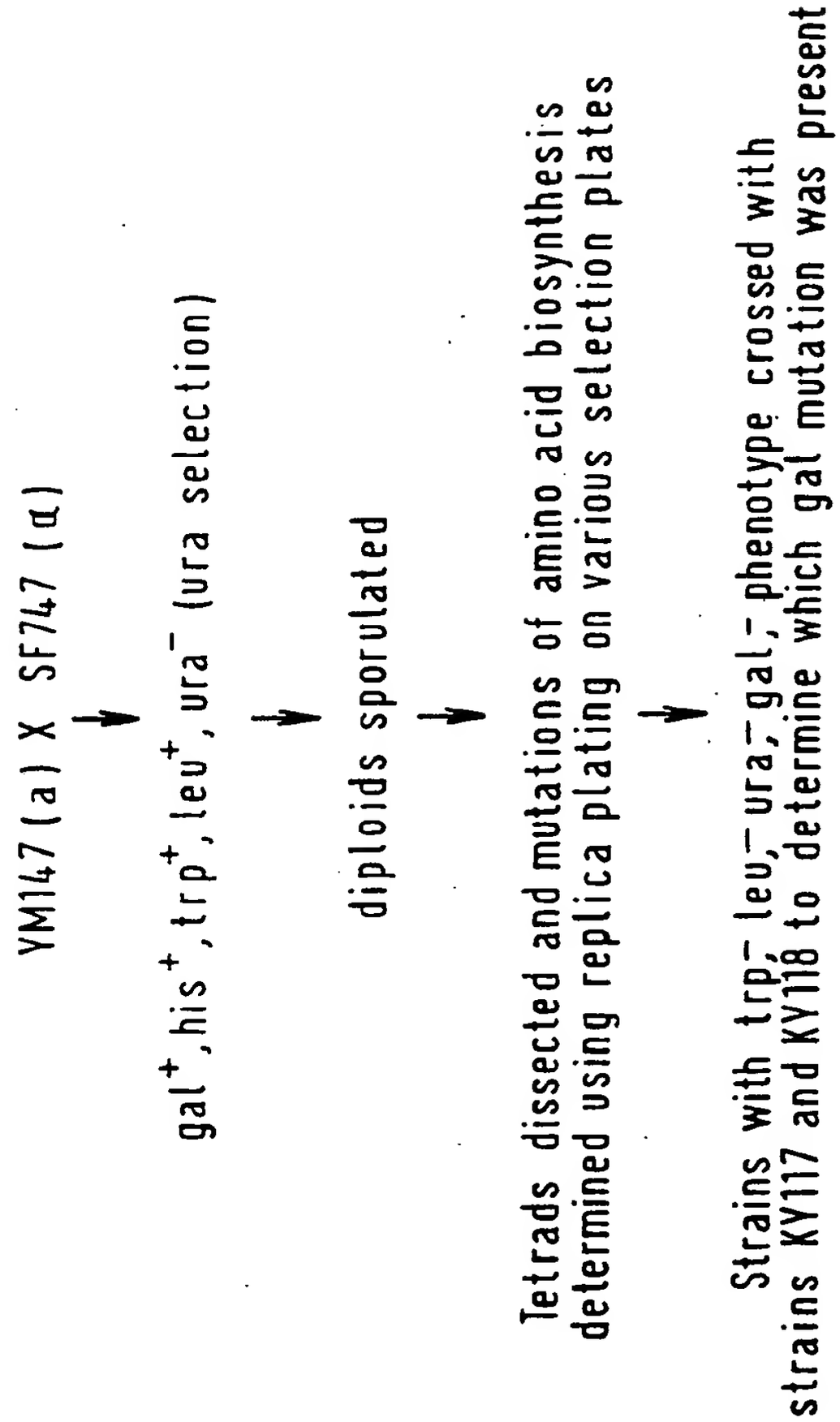

YM147 (a) X SF747 (α)
gal+, his+, trp+, leu+, ura− (ura selection)
diploids sporulated
Tetrads dissected and mutations of amino acid biosynthesis determined using replica plating on various selection plates
Strains with trp− leu− ura− gal− phenotype crossed with strains KY117 and KY118 to determine which gal mutation was present
Selection on galactose medium
BET9 (a)
BE27 (α)
his4, ura3, trp1, leu2, gal1
ura3, trp1, leu, gal1
Generation of a leu2, gal1 yeast strain.

FIG. 6.

BET9 UCP									
BET9 PKV									
BET9									
BE27 UCP									
BE27 PKV									
BE27									
	YPD YPD+Gal	YPDG YPDG+Gal	MMGlu MMGlu+Gal	MMLac MMLac+Gal	MMGly MMGly+Gal	LacCas LacCas+Gal	GlyCas GlyCasGal	2%Lac 2%Lac+Gal	

FIG. 7.

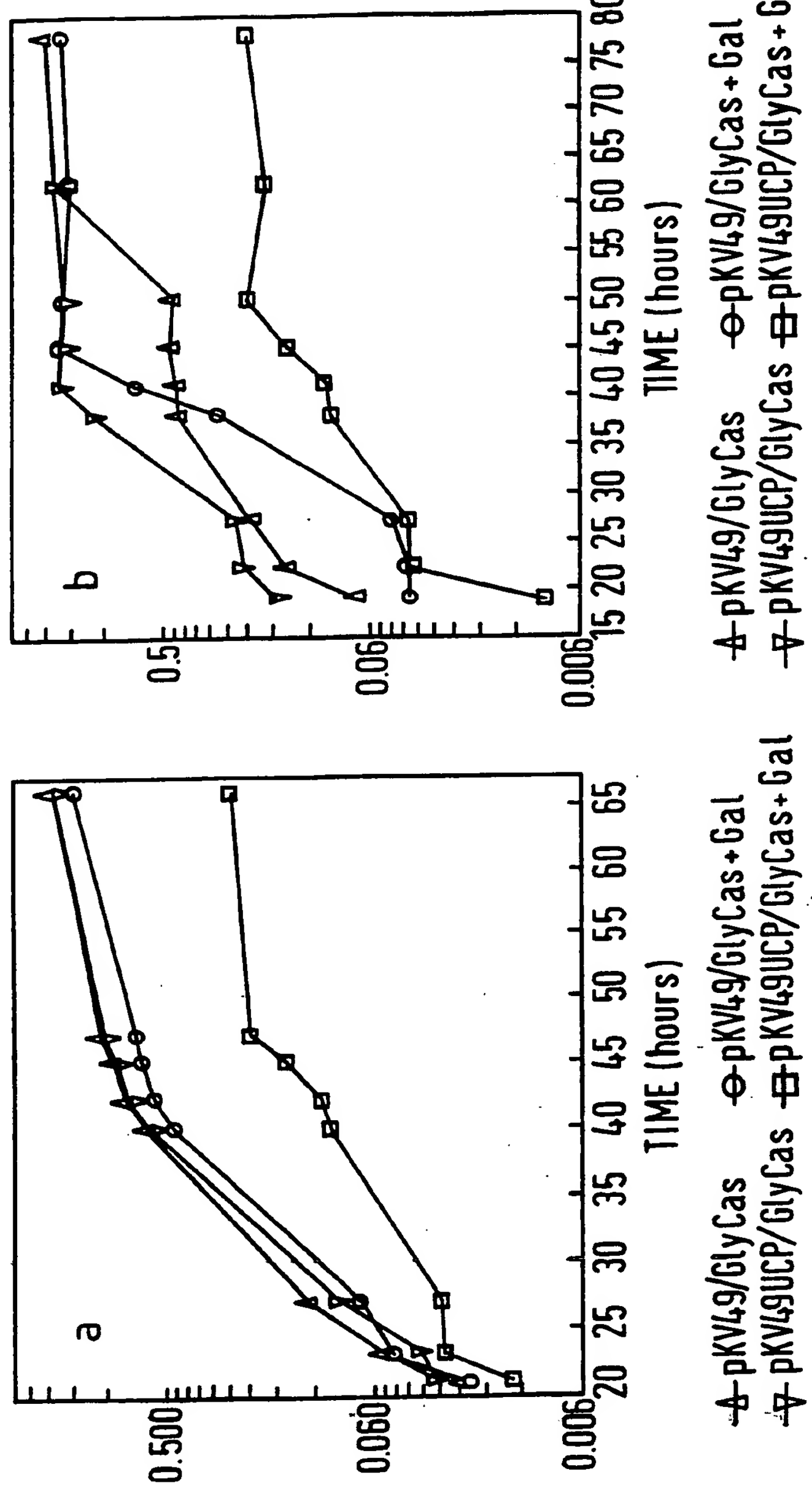

a
b
0.500
0.060
0.006
0.5
0.06
0.006
TIME (hours)
15 20 25 30 35 40 45 50 55 60 65 70 75 80
pKV49/GlyCas
pKV49UCP/GlyCas
pKV49/GlyCas+Gal
pKV49UCP/GlyCas+Gal

FIG. 8.

FIG. 9.

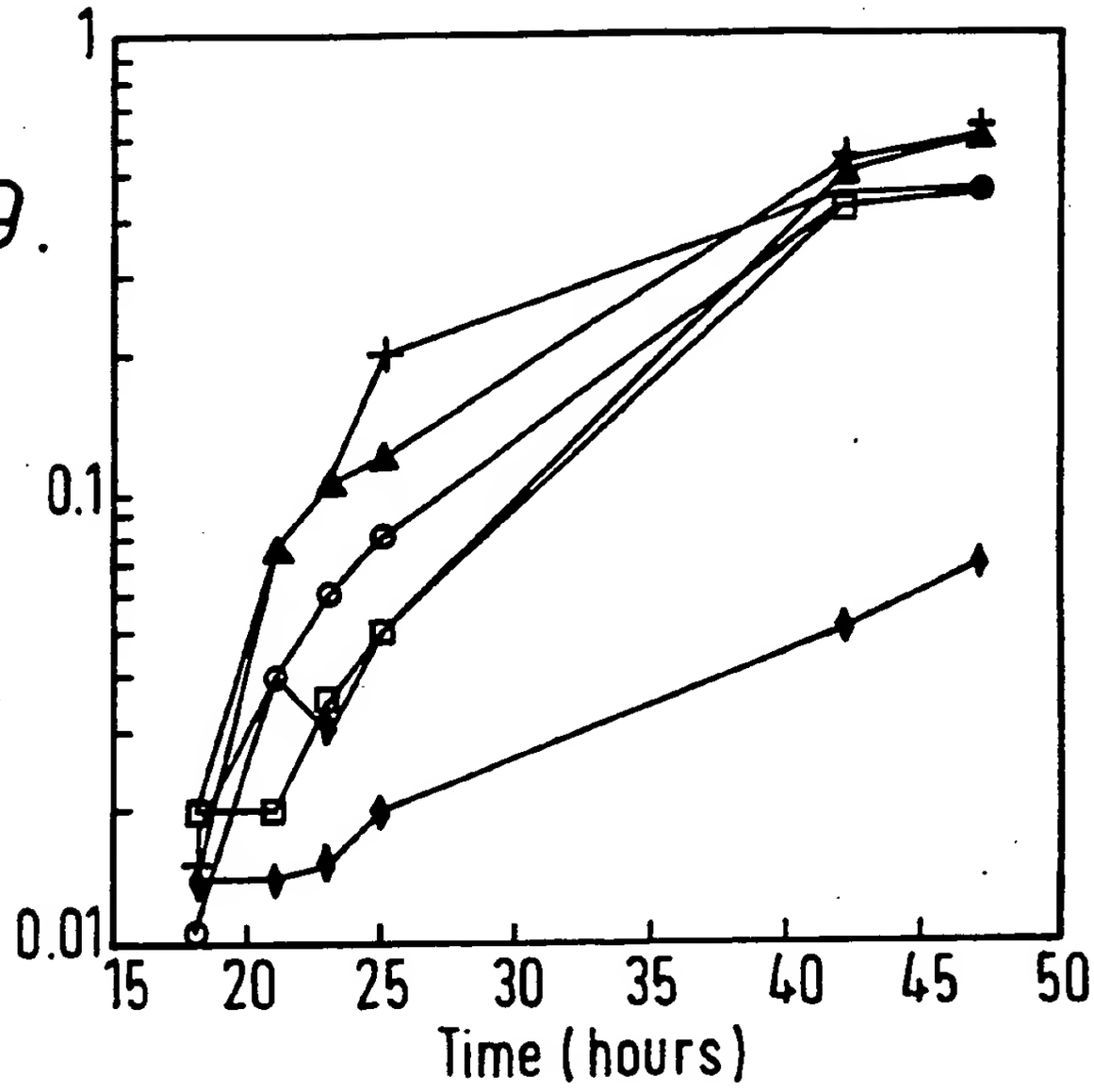
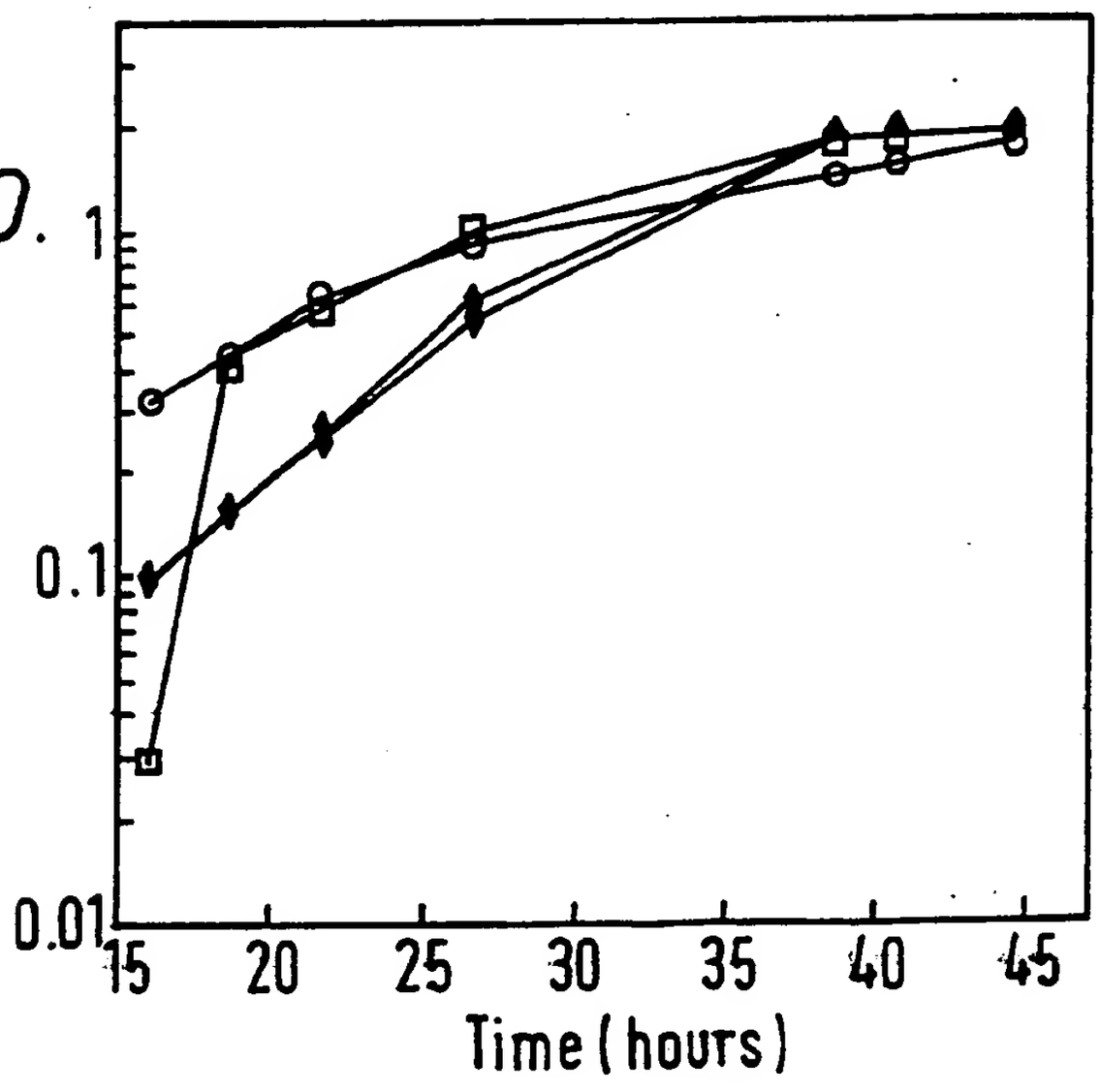

1
0.1
0.01
15
20
25
30
35
40
45
50
Time (hours)

FIG. 10.


1
0.1
0.01
15
20
25
30
35
40
45
Time (hours)

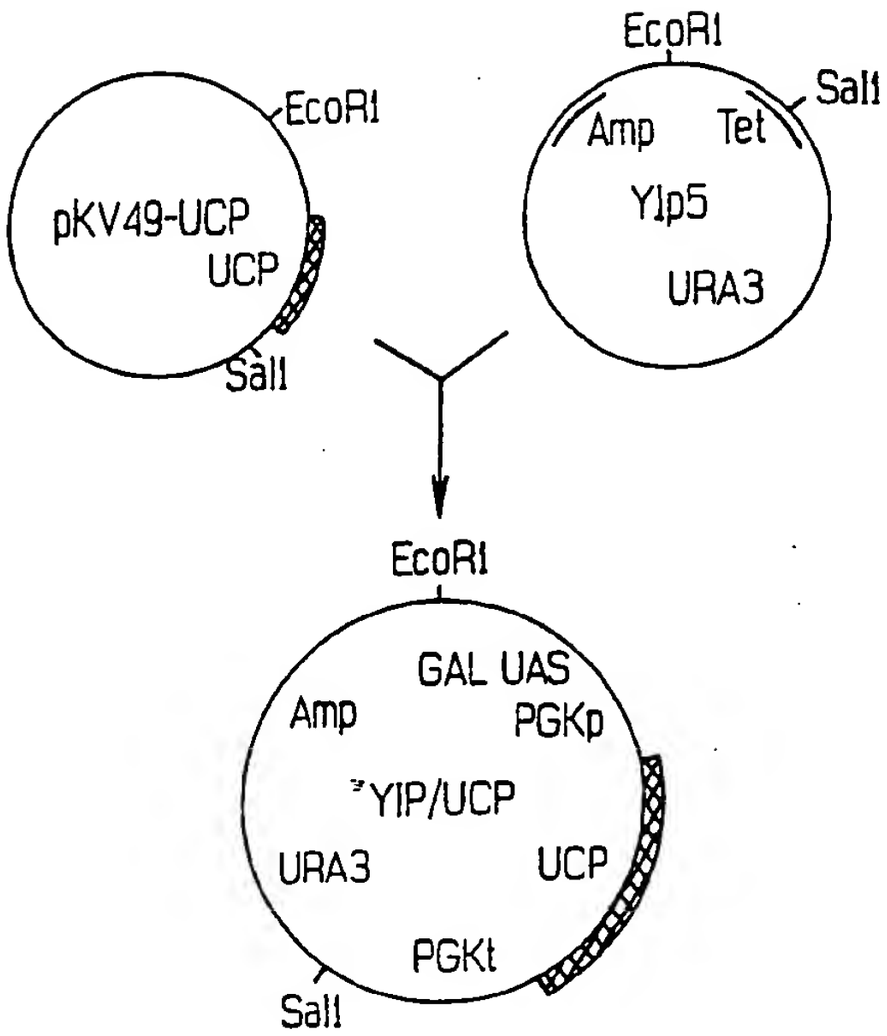
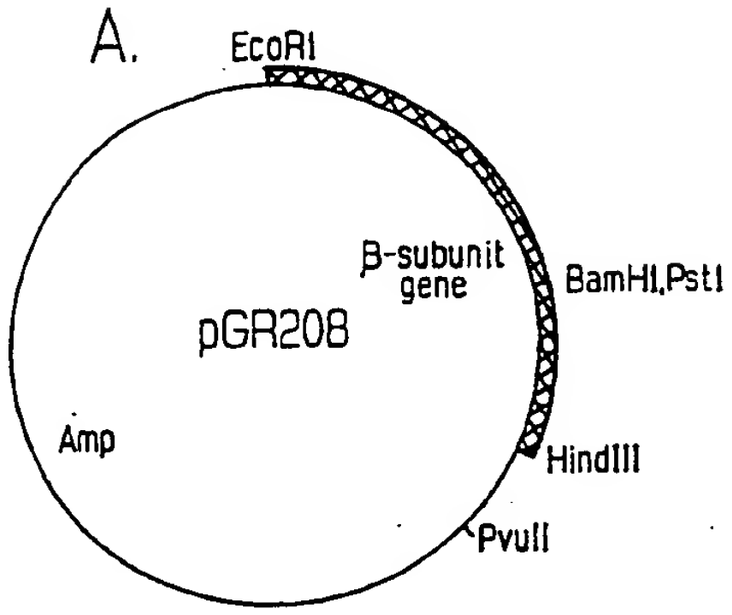


FIG. 11.

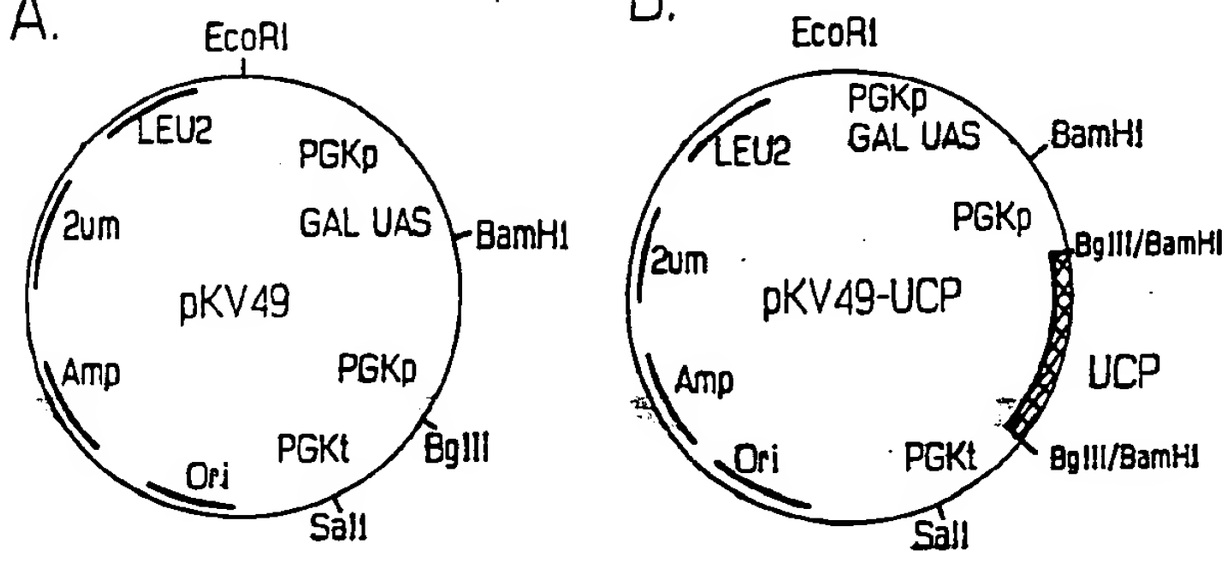
FIG. 12.



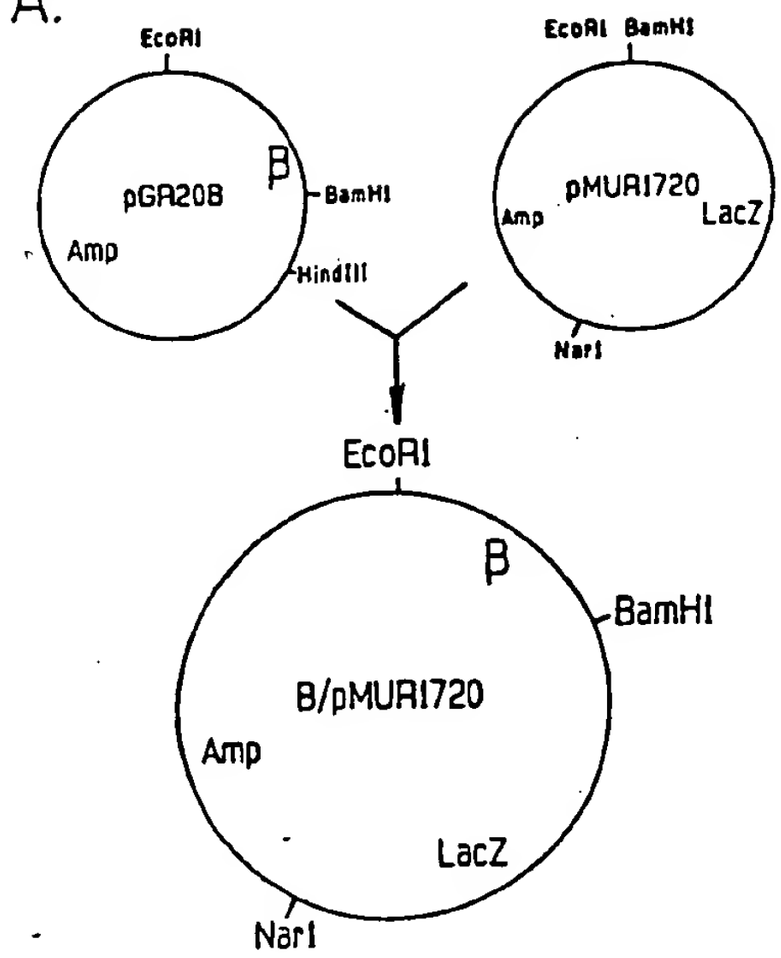
B.

Oligo B1	w.t.	ACGGTCAAATGAACGAACCTC
	mut.	ACGGTCAAATAAACGAACCTC
Oligo B2	w.t.	AGGTGTCGGTAAGACTGTGTT
	mut.	AGGTGTCGGTCAGACTGTGTT

FIG. 13.



A.



B.

Fusion Junction

ATT TAC CCT GCA GTG GAT CCC GTC GTT TTA

Ile	Tyr	Pro	Ala	Val	Asp	Pro	Val	Val	Leu
345	346	347	348	349	350		9	10	11

beto-subunit Loc-Z

FIG. 14. Construction of B-subunit / B-galactosidase fusion protein

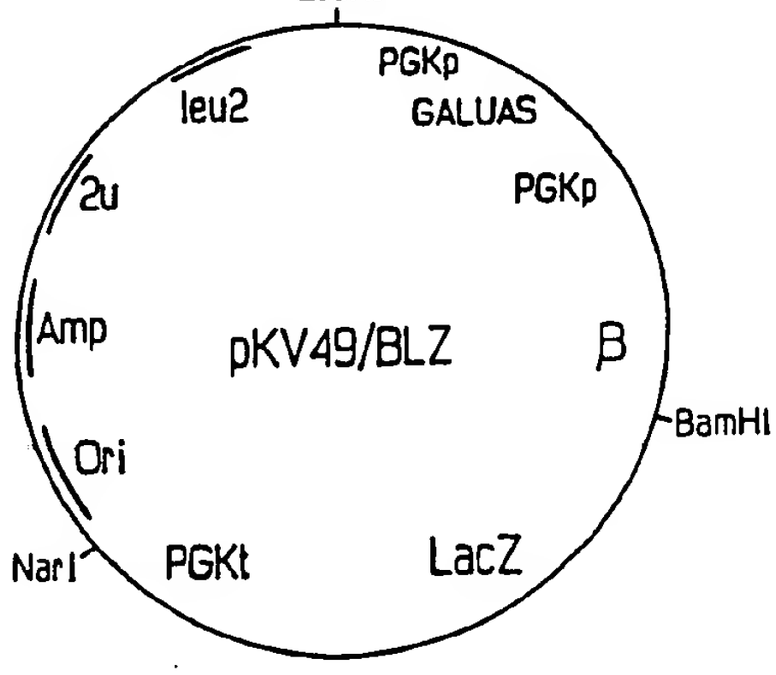
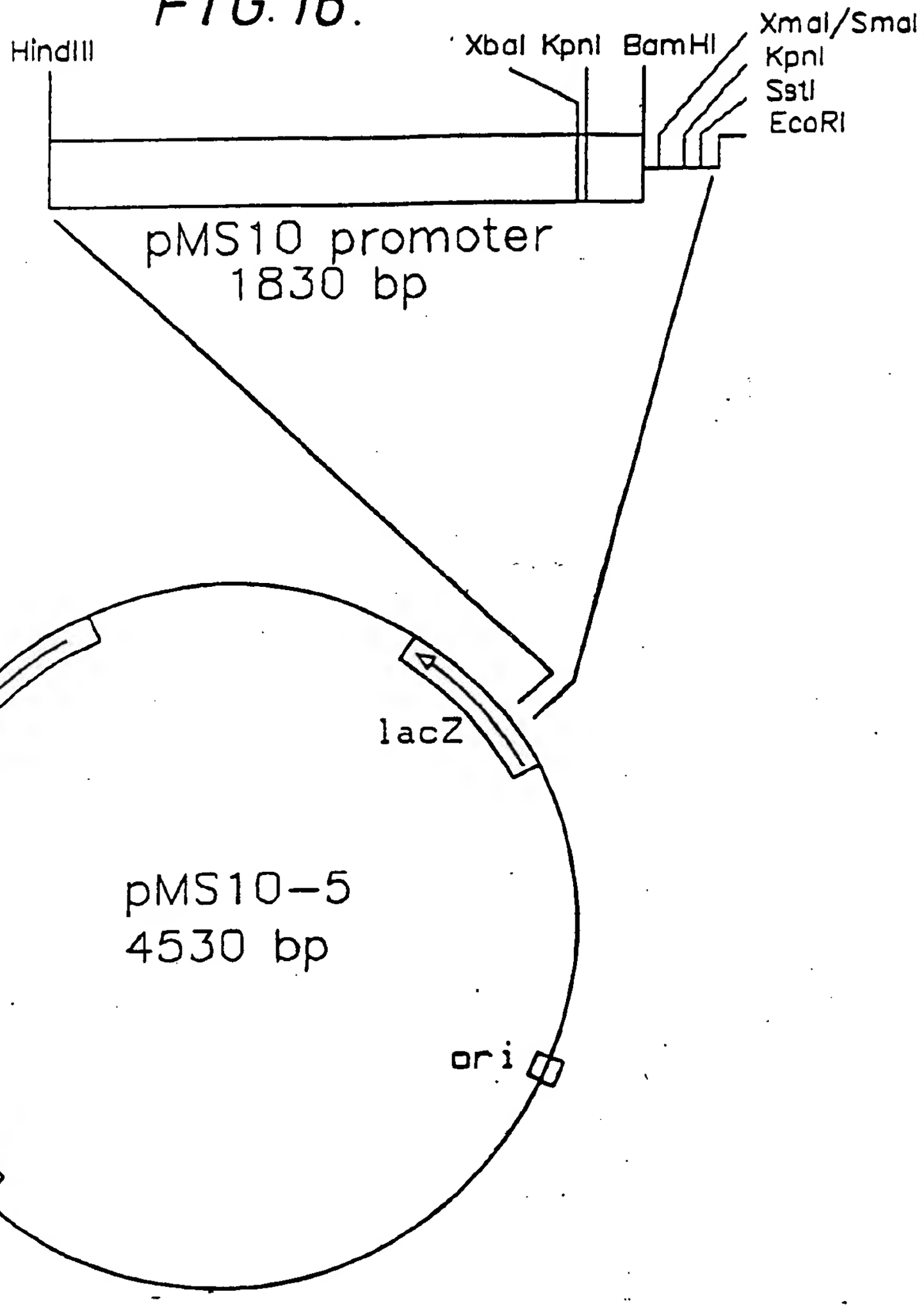


FIG. 15. Plasmid map of the construct pKV49/BLZ

FIG. 16.


HindIII
XbaI KpnI BamHI
XmaI/SmaI
KpnI
SstI
EcoRI
pMS10 promoter
1830 bp
lacZ
amp
pMS10-5
4530 bp
ori

16/16

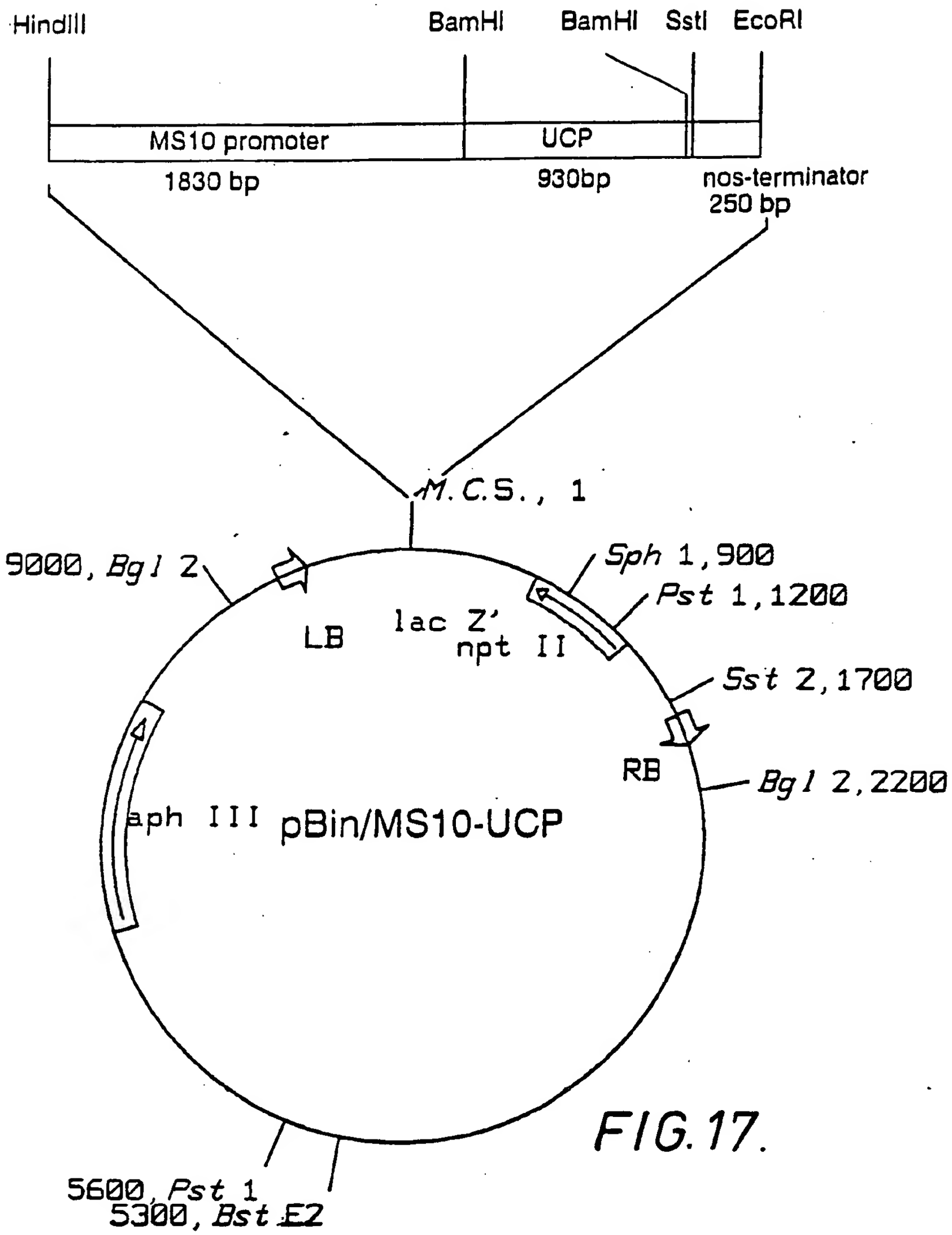

HindIII
BamHI
BamHI
SstI
EcoRI
MS10 promoter
1830 bp
UCP
930bp
nos-terminator
250 bp
M.C.S., 1
9000, Bgl 2
LB
lac Z'
npt II
Sph 1,900
Pst 1,1200
Sst 2,1700
RB
Bgl 2,2200
aph III
pBin/MS10-UCP
5600, Pst 1
5300, Bst E2

FIG. 17.

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 90/00114

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[5]: C 12 N 15/82, C 12 N 15/55, C 12 N 15/12

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System [8]	Classification Symbols
IPC[5]	C 12 N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [9]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	Cell, vol. 50, 3 July 1987, Cell Press E.G. Young et al.: "A fused mitochondrial gene associated with cytoplasmic male sterility is developmentally regulated", pages 41-49, see the whole article, particularly page 48, lines 1-5	11
Y	--	1
O,Y	Heredity, vol. 61, no. 2, 1988, 208th Meeting of the Genetical Society, Norwich, (GB), 13-15 April 1988, abstract 24 D.M. Lonsdale: "Chimeric genes associated with cytoplasmic male sterility" page 281, see abstract	1
	--	
P,X	WO, A, 89/10396 (PLANT GENETIC SYSTEMS NV) 2 November 1989, see pages 46-48 & EP, A, 0344029	1,7,11
	--	

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* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
3rd May 1990	05. 06. 90
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	MISS D.S. KOWALCZYK

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	EP, A, 0223247 (CIBA-GEIGY) 27 May 1987, see the whole document --	1
A	Nature, vol. 328, no. 6128, 23-29 July 1987, Basingstoke, Hampshire (GB) M. Boutry et al.: "Targeting of bacterial chloramphenicol acetyl transferase to mitochondria in transgenic plants", pages 340-342, see the whole article --	1
O,P, A	J. Cell. Biochem. Suppl. 13D Ucla Symposium on Plant Gene Transfer, 27 March - 7 April 1989, Alan R. Liss. Inc., New York (US) abstract M 310, M.B. Connett et al.: "Plant transformation as a test of the relationship between cytoplasmic male sterility, respiratory phenotype, and the PCF gene" page 299, see the abstract	1

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. GB 9000114 SA 34059

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 28/05/90
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A- 8910396	02-11-89	AU-A- 3537189	24-11-89
		EP-A- 0344029	29-11-89
EP-A- 0223247	27-05-87	AU-A- 6555586	28-05-87
		GB-A,B 2183660	10-06-87
		JP-A- 62155093	10-07-87